AMERICAN SOCIETY FOR MICROBIOLOGY | Clinical and Vaccine Immunology

Adenoviral Expression of a Bispecific VHH-Based Neutralizing Agent That Targets Protective Antigen Provides Prophylactic Protection from Anthrax in Mice

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***Bacillus anthracis*, the causative agent of anthrax, secretes three polypeptides, which form the bipartite lethal and edema toxins (LT and ET, respectively). The common component in these toxins, protective antigen (PA), is responsible for binding to cellular receptors and translocating the lethal factor (LF) and edema factor (EF) enzymatic moieties to the cytosol. Antibodies against PA protect against anthrax. We previously isolated toxin-neutralizing variable domains of camelid heavy-chain-only antibodies (VHHs) and demonstrated their *in vivo* efficacy. In this work, gene therapy with an adenoviral (Ad) vector (Ad/VNA2-PA) (VNA, VHH-based neutralizing agents) promoting the expression of a bispecific VHH-based neutralizing agent (VNA2-PA), consisting of two linked VHHs targeting different PA-neutralizing epitopes, was tested in two inbred mouse strains, BALB/cJ and C57BL/6J, and found to protect mice against anthrax toxin challenge and anthrax spore infection. Two weeks after a single treatment with Ad/VNA2-PA, serum VNA2-PA levels remained above 1 μg/ml, with some as high as 10 mg/ml. The levels were 10- to 100-fold higher and persisted longer in C57BL/6J than in BALB/cJ mice. Mice were challenged with a lethal dose of LT or spores at various times after Ad/VNA2-PA administration. The majority of BALB/cJ mice having serum VNA2-PA levels of >0.1 μg/ml survived LT challenge, and 9 of 10 C57BL/6J mice with serum levels of >1 μg/ml survived spore challenge. Our findings demonstrate the potential for genetic delivery of VNAs as an effective method for providing prophylactic protection from anthrax. We also extend prior findings of mouse strain-based differences in transgene expression and persistence by adenoviral vectors.**

Bacillus anthracis produces two toxins, which are responsible for allowing the bacterium to establish disease and induce lethality in the host. Lethal toxin (LT) and edema toxin (ET) are composed of three proteins: protective antigen (PA), lethal factor (LF), and edema factor (EF). PA is a receptor-binding component that transports LF (a protease) or EF (an adenylate cyclase) into cells where they can manifest their catalytic activities through the targeting of ubiquitous substrates. EF targets ATP and converts it to cyclic AMP (cAMP), resulting in cellular dysfunction and vascular events that can lead to lethality. LF cleaves the mitogen-activated protein kinase (MEK) family and rodent nucleotide-binding domain and leucine-rich repeat containing a pyrin domain 1 (NLRP1) inflammasome sensors. LF plays an important role in both early and late anthrax infection. Early in infection, inactivation of the MEK proteins by cleavage leads to the inhibition of a wide variety of innate immune cell responses, which allows the bacterium to evade the immune system, divide, and disseminate. The cleavage of NLRP1 early in infection in certain inbred rodents results in the activation of the inflammasome, macrophage pyroptosis, and induction of proinflammatory cytokines, which induce a protective immune response. Thus, certain inbred mouse strains are resistant to spore infection, while others are sensitive. Late in infection, high levels of both anthrax toxins in the blood induce unknown vascular events that contribute to the death of the host. The use of tissue-specific PA receptor knockout mice has now identified target tissues for both toxins. While the mechanism of LT-induced death is unknown, the cardiovascular system is clearly the important target, and PA acts as the "gateway" for all intoxication events (1).

PA is an 83-kDa polypeptide that binds to receptors expressed in most tissues. It is then cleaved by cell surface proteases, such as furin, to a 63-kDa form that rapidly oligomerizes. Heptamers or octamers of PA form binding sites for LF and EF (for a review, see reference 1). Because antibiotic treatment of *B. anthracis* infection is not effective after the anthrax toxins have accumulated in the blood, the targeting of PA is an important therapeutic approach against the disease. The majority of neutralizing antibodies against PA act on the receptor-binding domain 4 and prevent toxin interaction with cells. More rarely, PA is neutralized through other mechanisms (2).

Received 27 October 2015 **Returned for modification** 5 December 2015
Accepted 27 December 2015

Accepted manuscript posted online 6 January 2016

Citation Moayeri M, Tremblay JM, Debatis M, Dmitriev IP, Kashentseva EA, Yeh AJ, Cheung GYC, Curiel DT, Leppla S, Shoemaker CB. 2016. Adenoviral expression of a bispecific VHH-based neutralizing agent that targets protective antigen provides prophylactic protection from anthrax in mice. Clin Vaccine Immunol 23:213–218. doi:10.1128/CVI.00611-15.

Editor: P. P. Wilkins

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Alpacas, camels, and llamas are known to produce heavy-chain-only antibodies (for a review, see references 3 and 4). Variable domains of camelid heavy-chain-only antibodies (VHHs) can be expressed as recombinant proteins, which bind to antigen with affinity similar to that of the whole antibody (Ab), but they also have beneficial features, which include resistance to high temperature and pH and the ability to access conformational epitopes in folded structures, which are not generally reached by conventional antibodies (3, 4). Our laboratories have established the efficacy of VHHs against a variety of toxins (5–11). Linking of two or more neutralizing VHHs that target different epitopes creates VHH-based neutralizing agents (VNAs), which have proven to be greatly improved antitoxin agents compared to a pool of their component monomers (8–10, 12). We previously characterized a potent VNA for the treatment of anthrax (VNA2-PA), made as a heterodimer of two VHHs that neutralize PA by different mechanisms. One VHH, JKH-C7, inhibits the translocation of the cell surface-generated PA63 oligomer, while the other, JIK-B8, is a potent receptor blocker with a subnanomolar binding affinity for PA (6).

Gene therapy for *in vivo* expression of antibodies has had some success (13–17). In this work, we used a recombinant replication-incompetent human adenovirus serotype 5 (Ad5) vector that promotes expression and secretion into the serum of the VNA (Ad/VNA2-PA), thereby passively immunizing the mice. We measured antibody (Ab) levels over an 8-week period following a single bolus injection of Ad/VNA2-PA. We performed studies in two different inbred strains in parallel and found that robust protective Ab levels were rapidly established in both strains, but at significantly different levels, and then dissipated at different rates. Challenge studies done at various times posttreatment showed that mice having serum VNA levels of >1 μg/ml were protected from anthrax infection. Our results show the potential for VNA gene therapy as an anthrax therapeutic.

MATERIALS AND METHODS

Ethics statement. The animal studies were done in accordance with protocols approved by the animal care and use committees at the National Institute of Allergy and Infectious Diseases (NIAID) (protocols LPD8E and LPD9E).

Toxins. Endotoxin-free PA and LF were purified from *B. anthracis*, as previously described (18). LT is a combination of PA and LF, which are always used in equal amounts. The LF used here is a recombinant protein having an N-terminal sequence beginning with HMAGG. The LT concentrations correspond to the concentration of each toxin protein (i.e., 100 μg/ml LT is 100 μg/ml PA plus 100 μg/ml LF).

Spores. Spores were prepared from the nonencapsulated toxigenic *B. anthracis* Ames 35 (A35) strain (19) by growth on nutrient broth-yeast (NBY) sporulation agar at 37°C for 24 h, followed by 5 days at room temperature. Plates were inspected by microscopy to verify sporulation, spores were gently washed off with cold sterile water, and the plates underwent four additional cycles of sterile water washes and centrifugation. The preparations were then heat treated at 75°C for 1 h to kill any remaining vegetative bacteria. Spore quantification was performed using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA) and verified by dilution plating.

Ad/VNA2-PA construction and preparation. The generation of recombinant replication-incompetent Ad5-based vectors was previously described (20). Briefly, in a modification from the method of Mukherjee et al. (7), pShCMV-JGf7 shuttle plasmid was used for subcloning the VNA2-PA-coding sequence (6), under the control of the mammalian cytomegalovirus (CMV) promoter and followed by the bovine growth hormone poly(A) signal. A control vector, Ad/VNA-RT, was created in a similar manner with the sequence from two VHHs against ricin A chain (21). This control vector results in the secretion of a ricin-reactive Ab, with no binding to PA. Both shuttle plasmids were linearized and employed for homologous recombination with pAdEasy-1 plasmid, and the resultant plasmids containing viral genomes were validated by PCR, restriction analyses, and sequencing. The plasmids were linearized with PacI to release the inverted terminal repeats of the viral genomic DNA and transfected into 293 cells to rescue replication-incompetent Ad/VNA2-PA and Ad/VNA-RT. These Ad vectors were propagated in 911 cells, purified by centrifugation of CsCl gradients, and dialyzed, and the titers were determined.

Adenovirus and monoclonal administration and bleeds. C57BL/6J or BALB/cJ mice (female, 8 weeks old) were obtained from Jackson Laboratory (Bar Harbor, ME). Nonreplicative adenoviral vectors were diluted in sterile saline and injected intravenously (i.v.) in the tail (100 μl/mouse, 3×10^{10} or 1.2×10^{11} viral particles/study). In separate groups, BALB/cJ mice were injected with anti-PA monoclonal 14B7 (100 μg or 10 μg/mouse, i.v., 100 μl). Mice were bled by the mandibular or tail vein route at various days post-adenoviral vector administration, and serum was separated using serum separator tubes (Sarstedt, Newton, NC).

Serum VNA or monoclonal antibody measurement by ELISAs. Levels of the VNA2-PA or monoclonal 14B7 in serum were measured by enzyme-linked immunosorbent assay (ELISA). Immulon 2 HB immunoassay 96-well flat-bottom plates (Thermo Scientific, Franklin, MA) were coated with PA in phosphate-buffered saline (PBS) (10 μg/ml) overnight at room temperature. The plates were washed with PBS and blocked with 1% gelatin (100 μl/well; Bio-Rad, Hercules, CA) for 1 h. Serum from each mouse was serially diluted in triplicate, incubated for 2 to 3 h, removed, and washed 3 times with PBS-Tween (1× PBS plus 0.05% Tween 20). For sera from mice receiving adenoviral vector injections, a horseradish peroxidase (HRP)-conjugated anti-E-tag monoclonal antibody (MAb) (Bethyl Laboratories, Montgomery, TX) was added to each well at 1:3,000 dilution, incubated for 2 h, and washed 5 times with PBS-Tween. For mice receiving 14B7 injections, a higher percentage (1.3%) of Tween 20 was used in the washes, and an HRP-conjugated anti-mouse secondary antibody (Santa Cruz BT, Santa Cruz, CA) was used at 1:4,000. HRP substrate reagent (R&D Systems, Minneapolis, MN), made of a combination of stabilized hydrogen peroxide mixed with stabilized tetramethylbenzidine, was used for colorimetric assessment of HRP activity by spectrometry (450 nM). Purified VNA2-PA or 14B7 dilutions were used to construct standard curves, and Ab concentrations were calculated relative to these curves using the GraphPad Prism software.

Toxin and spore challenge. Mice were challenged with lethal doses of LT or spores at various times following adenovirus administration. Toxin challenges were performed in BALB/cJ mice, which are known to be LT sensitive but are spore resistant, due to harboring an LT-responsive *Nlrp1b* locus (22). Spore challenges were performed in the spore-sensitive C57BL/6J strain, which harbors a nonresponsive locus. LT (100 μg/mouse) was injected intraperitoneally (i.p.) (500 μl), while spores (5×10^7 A35 spores/mouse) were injected subcutaneously (s.c.) (200 μl) in the scruff of the neck. Mice were monitored for signs of malaise and survival twice daily for 7 days following infection.

RESULTS

We tested the efficacy of *in vivo* adenoviral production of heterodimeric anti-PA VNA2-PA by injecting BALB/cJ ($n = 15$) and C57BL/6J ($n = 15$) mice with 3×10^{10} viral particles of Ad/VNA2-PA and the same number of mice with a control adenoviral vector (Ad/VNA-RT) that produces an antiricin Ab not reactive to PA. The i.v. route was selected for the administration of vector, as earlier studies with a similar adenoviral vector expressing an anti-botulinum toxin VNA showed 6- to 7-fold-higher VNA levels following i.v. versus i.p. injections, and a benefit of >30-fold over the s.c. route (7). All mice were bled on day 10 and anti-PA VNA titers assessed. BALB/cJ mice had anti-PA VNA concentrations ranging

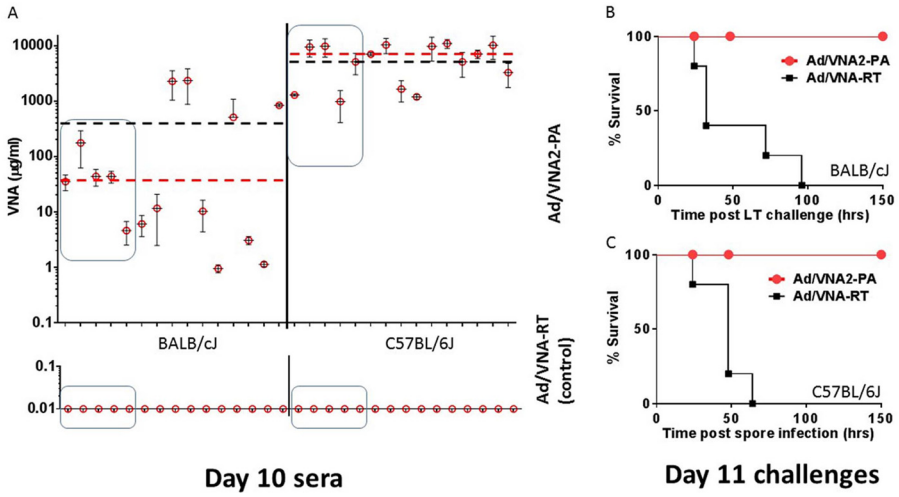

FIG 1 Day 10 serum analyses and day 11 challenge studies. (A) Groups of BALB/cJ or C57BL/6J mice (n = 15/group) were injected with Ad/VNA2-PA or Ad/VNA-RT (3×10^{10} viral particles), and VNA2-PA levels were assessed on day 10. Each circle refers to a single mouse, with the upper panel showing Ad/VNA2-PA groups and the lower panel showing the control vector groups. The dashed lines indicate the average (black) and median (red) Ad/VNA2-PA levels for each strain. The gray boxes indicate the mice that were challenged, with results shown in panels B and C. (B) BALB/cJ mice treated with Ad/VNA2-PA and control mice (n = 5/group; see panel A) were challenged on day 11 with LT (100 μg, i.p.) and monitored for malaise and survival. (C) C57BL/6J mice treated with Ad/VNA2-PA and control mice (n = 5/group; see panel A) were challenged with anthrax A35 spores (5×10^7 spores/mouse) and monitored for malaise and survival.

from 0.9 μg/ml to 2.3 mg/ml, with an average concentration of 419 μg/ml and a median concentration of 35 μg/ml. These levels were far lower than the 6.2 mg/ml average concentration (7.03 mg/ml median) measured for C57BL/6J mice (Fig. 1A). This is likely due to the fact that BALB/c inbred mice eliminate cells containing the Ad transgene much more rapidly than do C57BL/6 mice (23, 24). While 4 of 15 BALB/cJ mice had milligram per milliliter levels of VNA, the rest had levels of 1 to 100 μg/ml. The lowest level of VNA in C57BL/6J mice was 1 mg/ml, with the majority of mice having between 5 and 10 mg/ml VNA (Fig. 1A). Five mice from each strain were challenged in a blinded fashion on day 11. BALB/cJ mice were challenged i.p. with the 100% lethal dose (LD_{100}) of anthrax LT (100 μg), while spore-sensitive C57BL/6J mice were challenged with 5 LD_{100} of 5×10^7 spores. All mice treated with the Ad/VNA2-PA survived, while all challenged controls that had been treated with control vector succumbed (Fig. 1B and C).

VNA2-PA serum levels were again assessed on day 18 for all mice and were found to be reduced but to very different levels in a mouse strain-dependent manner. In BALB/cJ mice, 11/15 mice had levels of <1 μg/ml, while all C57BL/6J mice had titers of >10 μg/ml (Fig. 2A), as is discussed below. Because protection against

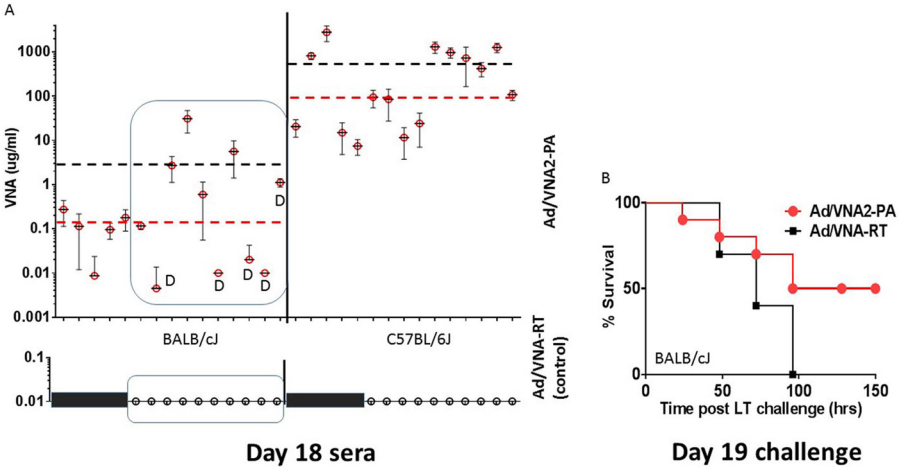

FIG 2 Day 18 serum analyses and day 19 challenge study results. (A) Day 18 sera from the mice that survived challenge and unchallenged mice described in Fig. 1 were analyzed for VNA2-PA levels. Each circle refers to a single mouse, with the upper panel showing Ad/VNA2-PA groups and the lower panel showing the control vector groups. The gray boxes indicate the two groups of 10 BALB/cJ mice that were challenged, with the results shown in panel B. The filled rectangles indicate the absence of sera for mice that succumbed in a previous challenge. The dashed lines indicate the average (black) and median (red) Ad/VNA2-PA levels for each strain. D, dead (5 mice that succumbed to the challenge described in panel B). (B) BALB/cJ mice treated with Ad/VNA2-PA and control mice (n = 10/group; see panel A) were challenged on day 19 with LT (100 μg, i.p.) and monitored for malaise and survival.

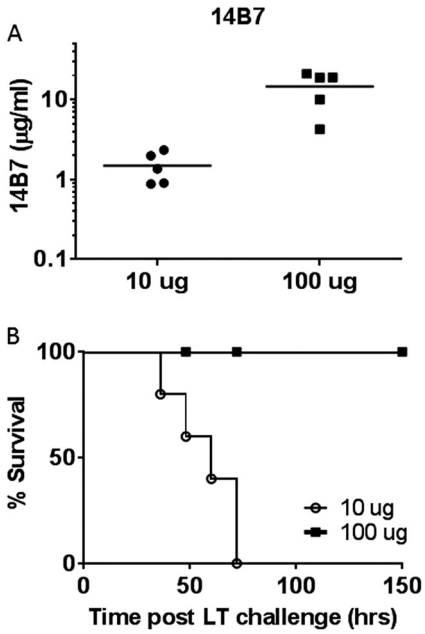

FIG 3 14B7 levels in sera and LT challenge outcome. (A) BALB/cJ mice (n = 5/group) were injected with either 10 or 100 μg of MAb 14B7 (i.v., 100 μl) and bled at 2 h to assess circulating levels of PA-specific monoclonal antibody. The solid line indicates the average MAb serum level. (B) All mice from panel A were challenged with LT (100 μg, i.p.) 2.5 h after MAb administration. The mice were monitored for a week for signs of malaise.

LT bolus challenge has historically required bolus administration of at least 50 μg of neutralizing MAb, we challenged the remaining BALB/cJ mice (n = 10) with the LD_{100} of LT on day 19. Of the 10 challenged mice, 50% survived challenge (Fig. 2B). It appeared that mice with VNA levels of >1 μg/ml (>18.6 nM) survived, with one exception, while mice with levels of <0.1 μg/ml (<1.8 nM) were not protected from this dose of toxin. Since VNA levels as low as ~10% (mol/mol) that of injected toxin were sufficient to save mice, this suggested that the majority of the toxin in the 100-μg mouse challenge had been cleared or processed or was not functionally relevant for lethality. Not surprisingly, all mice treated with control vector succumbed to toxin. To compare this protection threshold with that which is required for protection with the well-characterized anti-PA MAb 14B7, we assessed the levels of 14B7 in circulation after bolus challenge (Fig. 3A). A bolus of 100 μg of monoclonal 14B7 is fully protective (Fig. 3B), and this dose produced circulating MAb levels of 4 to 21 μg/ml (25 to 135 nM) when assessed at the time of toxin challenge, 2 h after MAb administration (Fig. 3A). A bolus of 10 μg/ml 14B7 was not protective (Fig. 3B), although levels of MAb in circulation ranged from 0.87 to 2.3 μg/ml (5.6 to 14.83 nM) at the time of challenge (Fig. 3A). It may be that VNA2-PA from gene therapy is slightly more efficient than 14B7 in toxin neutralization *in vivo*, possibly due to broader systemic distribution or access to tissue sites unavailable to 14B7. Overall, the protective threshold for an LD_{100} LT challenge when employing a potent antitoxin Ab appears to be 18 to 25 nM.

The C57BL/6J mice, which retained high levels of VNA2-PA at 18 days, were again monitored on days 25 and 33 post-Ad vector administration. There was a gradual drop in VNA levels over this period, with mice having a wide range of concentrations, from <0.1 to >10 μg/ml on day 33 (Fig. 4A). The control mice treated with Ad/VNA-RT had no detectable anti-PA VNA signal on day 25 or 33 (data not shown). When all the remaining unchallenged C57BL/6J mice (n = 10) were challenged with anthrax spores (5 LD_{100} [5×10^7 spores/mouse]) on day 34, there was a substantial delay in the onset of malaise and death in the mice treated with Ad/VNA2-PA, and 40% of challenged mice survived (Fig. 4B). Mice with VNA levels of <1 μg/ml (<18.6 nM) died. These results suggest that the levels of serum VNA2-PA antibodies that are protective in spore challenges of C57BL/6J mice are similar to the levels needed for the protection of BALB/cJ mice against lethal toxin challenge.

In a second study, we transduced BALB/cJ mice with a higher number of viral particles (1.2×10^{11} particles/mouse) and found that the levels of VNA2-PA were consistently higher than those in the first study, even at 2 weeks after vector administration, with all mice at >100 μg/ml and 3/16 mice at >1 mg/ml (Fig. 5). By 4 weeks, however, the levels of VNA for all mice had dropped pre-

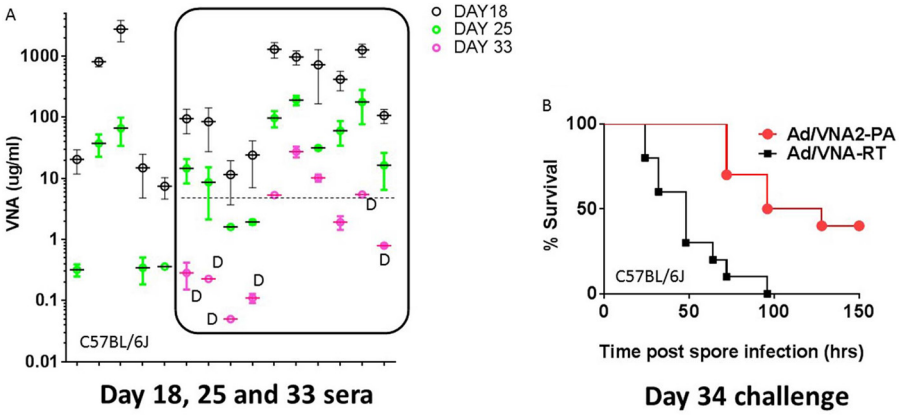

FIG 4 Day 25 and 33 serum analyses and day 34 spore challenge study results. (A) Comparison of day 18 serum VNA2-PA levels from Fig. 2 shown with levels on day 25 and 33. The dashed line indicates the average Ad/VNA2-PA levels for the 10 C57BL/6J mice on day 33. The box indicates the mice that were challenged on day 34. D, dead (6 mice that succumbed to the challenge described in panel B). (B) C57BL/6J mice treated with Ad/VNA2-PA and control mice (n = 10/group; see panel A) were challenged on day 34 with anthrax A35 spores (5×10^7 spores/mouse) and monitored for malaise and survival. Error bars indicate the standard deviation in multiple measurements from a single mouse.

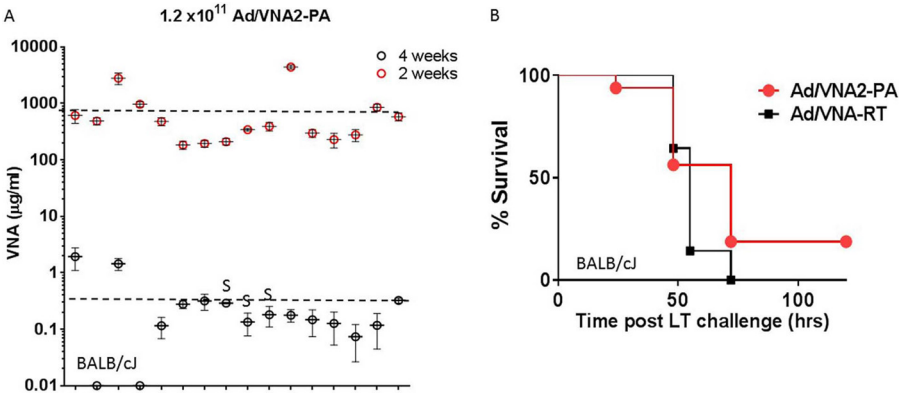

FIG 5 Study 2, week 2 and 4 serum analyses and week 5 LT challenge studies. (A) BALB/cJ mice ($n = 16$/group) were injected with Ad/VNA2-PA or Ad/VNA-RT (3×10^{10} viral particles), and VNA2-PA levels were assessed at 2 weeks and 4 weeks. The results shown are for the Ad/VNA2-PA group, as Ad/VNA-RT mice never had detectable PA-specific antibodies. The dashed lines indicate the average Ad/VNA2-PA levels at 2 weeks (top) and 4 weeks (bottom). Each circle refers to a single mouse. S, survived (3 mice that survived the challenge shown in panel B). (B) All mice in panel A were challenged at 5 weeks with LT (100 μg, i.p.) and monitored for malaise and survival. Error bars indicate the standard deviation in multiple measurements from a single mouse.

cipitously to the range of 0.1 to 1 μg/ml, indicating that the relative net loss of VNA in BALB/cJ mice occurred in proportion to the initial starting concentration but at a similar pace, independent of vector dose. Not surprisingly, only 3 of 16 mice challenged with LT survived.

DISCUSSION

We tested the ability of an Ad5-based adenoviral vector (Ad/VNA2-PA) expressing a bispecific VHH-based neutralizing agent (VNA2-PA) consisting of two linked VHHs targeting different anthrax toxin PA-neutralizing epitopes to protect mice against anthrax toxin challenge and anthrax spore infection. A single treatment with Ad/VNA2-PA resulted in antibody levels as high as 10 mg/ml. Levels were higher and persisted longer in C57BL/6J than in BALB/cJ mice. LT-sensitive BALB/cJ mice having serum VNA2-PA levels of >0.1 μg/ml typically survived LT challenge, and spore-sensitive C57BL/6J mice with levels of >1 μg/ml typically survived spore challenge. The studies presented here indicate that adenoviral delivery of VNAs can provide an excellent alternative to standard antibody therapeutics. Sustained levels of VNA with a single administration of nonreplicative Ad5 allows the host to combat the effects of toxin or virulence factors for longer periods than repeated administration of purified antibody. Furthermore, the gene therapy vectors can be used as prophylactic therapeutics if there is a danger of exposure of large populations to toxic agents. The VHH-based therapeutics can also be engineered to make VNAs that target multiple toxins or agents in a single product, delivered with a single inoculation (9). These vectors also allow the possibility to deliver VNAs to specific tissue sites through vector engineering (25). While Ad5 vector use in humans might be limited by widespread preexisting immunity, alternative gene therapy vectors, such as adenoviruses from simian sources or adeno-associated viruses, are being developed and may prove more practical for general use.

Interestingly, the levels of VNA in our current studies were not sustained for as long a period as those observed for a similarly constructed anti-botulinum antitoxin VNA, which was delivered i.v. at 3×10^{10} viral particles into CD-1 Swiss mice (7). In that study, VNA levels remained at 1 to 10 mg/ml in half the mice, even at 8 weeks postinoculation, although some mice had significantly lower levels as early as 10 days postinoculation. Thus, the range of serum VNA might vary from 0.01 ng/ml to >100 μg/ml in the mice at 6 to 8 weeks after vector administration. The reason for this is very likely the genetic heterogeneity of CD-1 mice, which are outbred. Genetic factors have been reported to influence the efficiency of Ad infection and/or transgene production (24, 26, 27), and it is not surprising that these factors would be more varied in the outbred mice than observed within the two inbred lines used in the current study. The same genetic factors are expected to exist among humans and thus lead to differential responses to various forms of antibody and VNA gene therapy.

Our findings that protection against anthrax toxin is possible for weeks after a single administration of Ad/VNA2-PA suggest that adenoviral antianthrax therapeutics are a viable option as future therapeutic agents against this disease. The options to administer intranasal Ad/VNA2-PA vectors or administer parenteral Ad/VNA2-PA vectors designed to promote pulmonary VNA expression (28) may result in even more effective therapeutics for anthrax exposures. Future studies will focus on tissue-specific targeting of VNA gene therapy vehicles for more efficient neutralization of toxic effects at relevant disease sites.

ACKNOWLEDGMENT

We thank the staff of the NIAID Comparative Medicine Branch for assistance with vivarium studies.

FUNDING INFORMATION

HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Charles B. Shoemaker under grant number U54 AI057159. HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Stephen Leppla under grant number 1ZIAAI000929-12.

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